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Antisense Oligonucleotide-mediated Exon Skipping as a Systemic Therapeutic Approach for Recessive Dystrophic Epidermolysis Bullosa

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The "generalized severe" form of recessive dystrophic epidermolysis bullosa (RDEB-gen sev) is caused by bi-allelic null mutations in *COL7A1*, encoding type VII collagen. The absence of type VII collagen leads to blistering of the skin and mucous membranes upon the slightest trauma. Because most patients carry exonic point mutations or small insertions/deletions, most exons of *COL7A1* are in-frame, and low levels of type VII collagen already drastically improve the disease phenotype, this gene seems a perfect candidate for antisense oligonucleotide (AON)-mediated exon skipping. In this study, we examined the feasibility of AON-mediated exon skipping *in vitro* in primary cultured keratinocytes and fibroblasts, and systemically *in vivo* using a human skin-graft mouse model. We show that treatment with AONs designed against exon 105 leads to in-frame exon 105 skipping at the RNA level and restores type VII collagen protein production *in vitro*. Moreover, we demonstrate that systemic delivery *in vivo* induces *de novo* expression of type VII collagen in skin grafts generated from patient cells. Our data demonstrate strong proof-of-concept for AON-mediated exon skipping as a systemic therapeutic strategy for RDEB.

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Introduction

Recessive dystrophic epidermolysis bullosa, generalized severe (RDEB-gen sev; OMIM# 226600) is a devastating skin blistering disease. The disease is caused by bi-allelic null mutations in the *COL7A1* gene encoding type VII collagen.[1] Type VII collagen is the major component of anchoring fibrils that secure attachment of the epidermis to the dermis and is expressed by both basal epidermal keratinocytes and dermal fibroblasts.[2] The absence of type VII collagen in RDEB-gen sev leads to severe blistering of the skin and mucosa just below the lamina densa. Abnormal wound healing with excessive scarring inevitably results in the fusion of fingers and toes (*i.e.*, pseudosyndactyly).[3] Patients have a highly increased risk of developing aggressive squamous cell carcinomas, which is the major cause of death before the age of 30–40 years.[4] The *COL7A1* gene comprises 118 small exons that encode the type VII collagen pro-α1 chain, which consists of a central 145 kDa triple helix domain (THD) flanked by a 145 kDa amino-terminal non-collagenous 1 (NC1) domain and a 30 kDa carboxyl-terminal non-collagenous 2 (NC2) domain.[5] Post-translational modification leads to stable trimerization of three pro-α1 chains to pro-type VII collagen homotrimers, followed by partial removal of the NC2 domain and antiparallel dimerization of type VII collagen trimers.[6] Numerous type VII collagen dimers aggregate laterally to form anchoring fibrils that attach the epidermis to the dermis. Notably, all exons that encode the triple helix are in-frame and most encode repetitive glycine-X-Y amino acid sequences, where X and Y can be any amino acid.

At the moment, treatment for RDEB-gen sev is merely symptomatic. Several therapeutic approaches have been studied,[7–12] however, there still is a great need for novel and, highly preferably, systemic approaches. Antisense oligonucleotide (AON)-mediated exon skipping seems to be an attractive therapeutic approach for RDEB-gen sev. In this approach, short modified RNA molecules (e.g., 2'-*O*-methyl phosphorothioates, locked nucleic acids, or phosphorodiamidate morpholinos) are designed to modulate pre-mRNA splicing of specific in-frame target exons harboring the disease-causing mutation. Through complementary binding of the AON to the target exon, the exon is hidden from the splicing machinery and spliced out with its flanking introns, bypassing the mutation and allowing the production of an internally deleted, but in the ideal outcome, functional protein.[13] *COL7A1* is a good candidate gene for AON-mediated exon skipping, as most RDEB-gen sev patients have small exonic mutations, and most *COL7A1* exons are in-frame and encode highly repetitive Gly-X-Y amino acid stretches. This is underscored by findings that patients carrying *COL7A1* mutations that lead to natural skipping of an in-frame exon have relatively mild phenotypes.[14,15] Additionally, the severity of the clinical phenotype in RDEB is highly correlated to the level of expression of type VII collagen at the cutaneous

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basement membrane zone (BMZ); the slightest increase in type VII collagen deposition at the BMZ already leads to a marked improvement in clinical phenotype.[16]

Pioneer attempts to induce exon skipping in *COL7A1* have been described before: Turczynski *et al.* induced exon skipping *in vitro*, while Goto *et al.* were able to induce localized exon 70 skipping *in vivo*.[17,18] However, the severe multi-organ involvement in RDEB demands a generalized treatment approach, which renders systemic delivery of AONs crucial to therapeutic success. In this study, we have therefore taken the exon skipping approach a leap forward to clinical application by demonstrating the potential of systemic delivery of the AONs using an *in vivo* grafting model.

Results

Selection of exons eligible for exon skipping

To study the applicability of exon skipping in the *COL7A1* gene, all 118 exons of *COL7A1* were analyzed *in silico* (summarized in **Figure 1**, NCBI reference sequence: NM_000094.3). Out of 118 exons, 107 are in-frame, *i.e.*, more than 90%. The THD of type VII collagen protein is encoded solely by in-frame exons and consists of 84 exons that collectively encode 454 Gly-X-Y amino acid sequence repeats. Interestingly for exon skipping, 60 of these 84 exons encode perfect Gly-X-Y sequence motifs only, ranging from 3–13 Gly-X-Y triplets (collectively 337/454 triplets). Moreover, the reading frame of all exons in the THD start at position 1 and end at position 3. Hence, skipping of one of these exons will not result in an amino acid change at the skipping junction and leave the Gly-X-Y repeat structure intact. The Gly-X-Y sequence is essential for triple helix formation, however, the length of the triple helix is not essential for its function.[19] Therefore, it is predicted that skipping of an exon encoding a Gly-X-Y sequence only will have the least functional consequences. The in-frame nature of the 107 exons, the Gly-X-Y repeat structure, and the reading frame, makes **Figure 1** a roadmap for exon skipping therapy in the *COL7A1* gene.

For this study, exon 105 was chosen as a target, as primary keratinocyte and fibroblast cultures were readily available from patient EB-023 suffering from RDEB-gen sev due to the homozygous nonsense mutation c.7828C>T, p.Arg2610Ter in exon 105. This nonsense mutation introduces a premature termination codon (PTC), which induces nonsense-mediated mRNA decay (NMD), resulting in the total absence of type VII collagen in the patient's skin explaining the severe phenotype (**Supplementary Figure S1**). Additionally, exon 105 is an 81 bp in-frame exon that encodes nine Gly-X-Y repeats, and, as such, skipping of exon 105 is predicted to be tolerated.

In vitro exon skipping results in restoration of type VII collagen synthesis

According to published data on AON design,[12] all 1,134 possible 17–23 base pair long sequences in the region of exon 105, were analyzed *in silico* for their Tm (>48 °C), GC-content (40–60%), binding energy (15–30), and off target binding. Splice enhancer sequences in and around exon 105 were visualized using prediction software.[20] The two most promising sequences were synthesized as 2'-*O*-methyl phosphorothioate AONs and analyzed for their exon skipping abilities (**Figure 2a**). PCRs using primers spanning exon 102–108 were used to assess exon skipping and possible nearby upstream or downstream splice effects. Optimization of the *in vitro* transfection experiments in control cells showed that the highest exon-skipping efficiency was achieved using a combination of both AONs in a total concentration of 250 nmol/l (**Figure 2b**). This combination was used in further experiments.

Control and patient primary keratinocytes and fibroblasts were subsequently cultured and transfected with the combination of the two specific exon 105 AONs or non-specific AONs. Forty-eight hours after transfection, RNA was isolated from transfected control and patient keratinocytes and fibroblasts. In the control and patient cells transfected with the exon 105 AONs, RT-PCR analysis revealed exon skipping with a high efficiency at the RNA level in both control and patient keratinocytes and fibroblasts. Exon 105 deletion was confirmed by Sanger sequencing (**Figure 2c**).

To assess *de novo* expression of type VII collagen resulting from exon 105 skipping, control and patient cells were cultured on cover slips prior to transfection with the two exon 105 AONs. Seventy-two hours after transfection, the cells were fixated to the cover slips and analyzed by

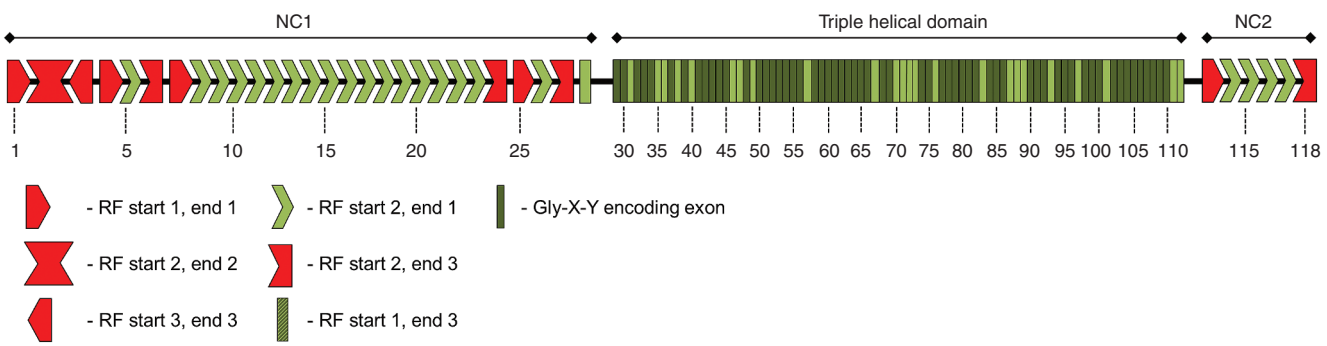

Figure 1 Road map for exon skipping in the *COL7A1* gene. The figure represents all *COL7A1* exons and their corresponding reading phases. The shapes of the exons depict the phasing of the triplet codons over different exons. Light and dark green boxes indicate skippable exons, which can be divided into two groups: those that start and end with a complete codon (square boxes), all but one located in the triple-helix domain (THD) domain, and those that begin and end with a partial codon (arrow shaped boxes), located exclusively in the NC1 and NC2 domains. Red boxes depict unskippable exons. Exons 29 to 112 encode the collagenous THD. Dark green boxes depict exons of the THD that encode a Gly-X-Y amino acid sequence only.

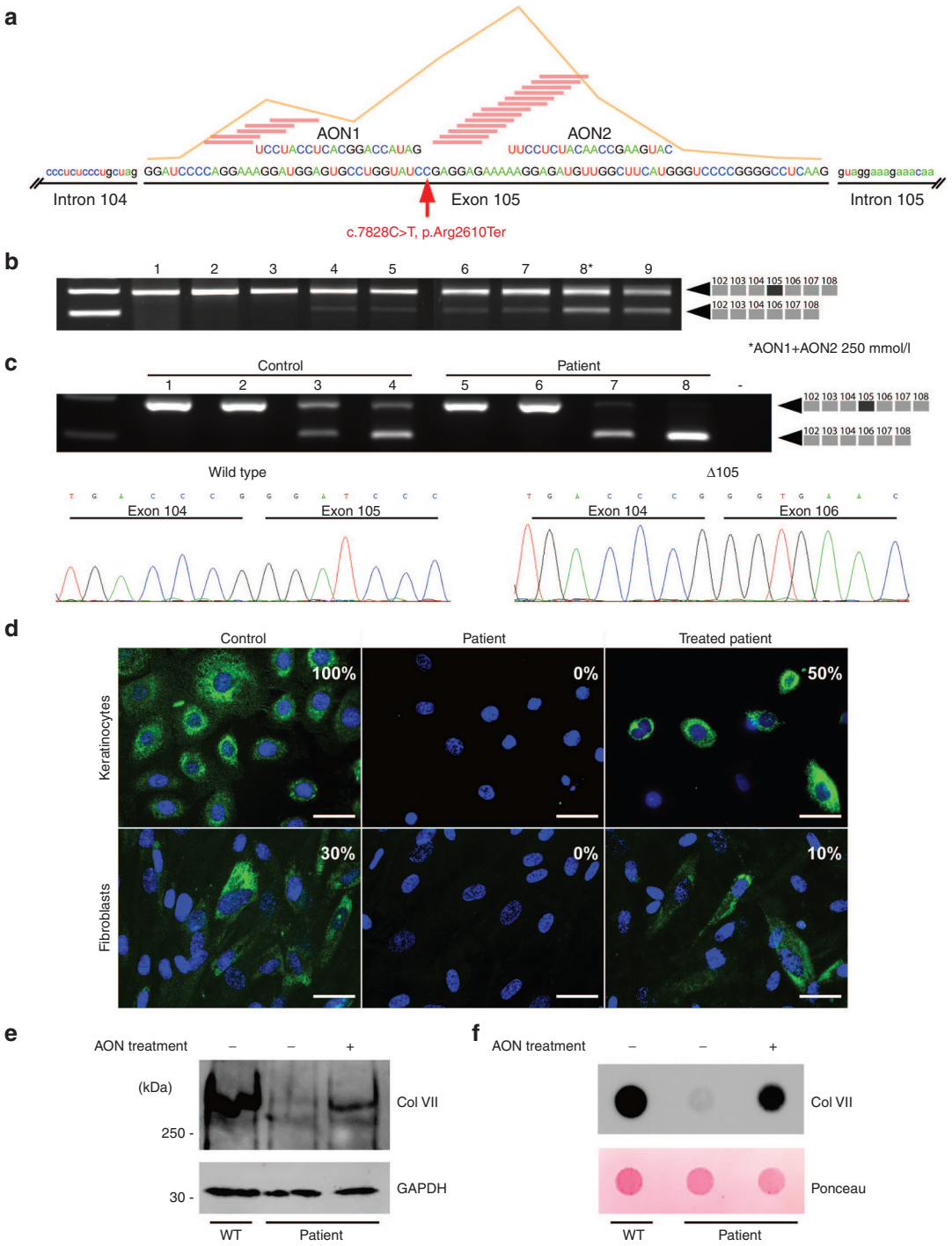

Figure 2 Specific antisense oligonucleotides (AONs) restore type VII collagen synthesis *in vitro*. (**a**) Position of AON1 and AON2 and patient's mutation (red arrow) in exon 105. Predicted exon splice enhancer sequences reveal two potential regions for AON targeting (pink bars and orange curve). (**b**) RT-PCR on patient keratinocytes showed most effective exon skipping with 250 mmol/l of both AONs. Healthy keratinocytes (1), Scrambled AONs (2–3), 250 mmol/l and 500 mmol/l AON1 (4–5), AON2 (6–7) or AON1+AON2 (8–9). (**c**) RT-PCR on healthy and patient keratinocytes and fibroblasts after transfection with 250 mmol/l AONs (3–4 and 7–8 respectively) or scrambled AON (1–2 and 5–6 respectively). Lower panel shows confirmation of exon 105 skipping by Sanger sequencing. (**d**) Cells immunostained for type VII collagen treated or untreated with 250 mmol/l AONs (percentage represents the number of type VII collagen expressing cells). Scale bar = 15 μm. (**e**) Western blot analysis on healthy control, untreated patient, and treated patient keratinocyte cell lysates, reveals the expression of type VII collagen by transfected patient keratinocytes. (**f**) Dot blot analysis on conditioned medium reveals that the newly formed Δ105 type VII collagen can be secreted. N.B. Due to overexpression of the membrane, and the use of a polyclonal antibody, the blots in **e** and **f** showed minor residual staining in untreated patient cells, whereas no staining was observed with LH7.2 monoclonal antibody in **d**.

immunofluorescence (IF) staining. In contrast to control keratinocytes and fibroblast, no expression of type VII collagen was observed in patient cells, either untransfected or transfected with a nonspecific AON. However, when patient keratinocytes and fibroblasts were transfected with the specific AONs against exon 105, a distinct *de novo* expression of type VII collagen was observed (**Figure 2d**). Compared to healthy control cells, restoration of type VII collagen expression was observed in 50 and 33% of transfected patient keratinocytes and fibroblasts, respectively. Restoration of protein synthesis was calculated by examining 1,500 cells for type VII collagen expression for each transfection condition.

Western blot analysis on protein lysates from patient cells treated with AONs for 72 hours showed that treated patient cells synthesized the full length of the exon 105 deleted type VII collagen. Moreover, the level of type VII collagen restoration was 14% compared to healthy control cells (**Figure 2e**). Further, dot blot analysis on conditioned medium revealed that the newly synthesized type VII collagen lacking the amino acids encoded by exon 105 could be secreted by the transfected patient cells (**Figure 2f**).

Systemically induced restoration of type VII collagen expression *in vivo*

To establish preclinical relevance, the AONs were tested in an *in vivo* model.[21] To this end, we reconstituted skin grafts of primary cultured patient fibroblasts and keratinocytes on the back of athymic immune-deficient nude mice (**Figure 3a**, **Supplementary Figure S2**). IF staining specifically for human type VII collagen showed brightly positive staining of the BMZ in the human skin graft and negative staining in mouse skin. Thus, this skin graft model represents a personalized mouse model offering the opportunity to easily and directly test the *in vivo* efficacy of AONs on patient skin and additionally allows long-term treatment and observation of treatment effect on the target skin.

Six mice were grafted with patient keratinocytes and fibroblasts carrying the premature termination codon c.7828C>T;p.Arg2610Ter mutation in exon 105, and two mice were grafted with healthy control keratinocytes and fibroblasts. During the treatment phase, four out of the six mice bearing patient skin grafts were treated with five times a week 50 mg/kg of each AON (100 mg/kg in total) via subcutaneous injections at the tail base, *i.e.*, approximately 7 cm distal from the skin grafts, for a period of 8 weeks (injection site indicated in **Figure 3a**). The two remaining mice bearing patient skin grafts, and the two mice bearing healthy control skin grafts were given subcutaneously injected saline solution as a negative control.

The total AON dose of 100 mg/kg was used because previous pharmacodynamics and pharmacokinetics studies for Duchenne muscular dystrophy (DMD) showed saturation of serum protein binding beyond this concentration.[22] The choice for the subcutaneous administration route was based on our positive experience with a DMD mouse model (*mdx*) where dystrophin exon skipping could be detected in skin samples (data not shown), and the absence of differences in bioavailability of 2'-*O*-methyl phosphorothioates injected either subcutaneously or intravenously.[22]

After 8 weeks of treatment, the human skin grafts were harvested and RNA was isolated from graft cryosections. Subsequent RT-PCR analysis revealed exon 105 skipping in the RNA isolated from the patient grafts grown on mice treated with the specific exon 105 AONs. Sanger sequencing confirmed exon 105 skipping (**Figure 3b**). As expected, exon 105 skipping was not observed in RNA isolated from the patient or control grafts grown on mice injected with saline solution.

Further, human skin graft cryosections were immunostained for human type VII collagen, which revealed bright staining at the BMZ in control graft sections treated with saline and complete absence of type VII collagen expression in patient graft sections treated with saline. In contrast, clear *de novo* expression of type VII collagen at the BMZ was evident in patient graft sections isolated from mice treated systemically with the specific exon 105 AONs (**Figure 3c**). The staining intensity of type VII collagen varied along the BMZ and the overall amount of type VII collagen expression was lower than in the control graft sections, however, restoration of type VII collagen expression was unequivocally observed. Immunostaining for type IV collagen in untreated patient skin grafts revealed typical RDEB basement membrane abnormalities with clear widening of the BMZ and off-shoots deep into the papillary dermis (**Figure 3d**). Interestingly, such basement membrane deformities were neither observed in the treated patient skin grafts, nor in the healthy control grafts. These results do not only show efficacy of the AON treatment, but also advocate the functionality of the *de novo* Δ105 type VII collagen protein expressed in treated patient skin grafts.

Discussion

Several therapeutic strategies for RDEB are being investigated at cell, protein, RNA, or DNA level.[23,24] For example, the injection of type VII collagen expressing fibroblasts,[25] induced pluripotent stem cells as a source for the regeneration of healthy skin,[8,26] placenta-derived stem cell strategies,[27] and *ex vivo* gene editing.[12] Another group of promising therapeutic approaches is acting either through or on RNA transcripts, e.g. translational premature termination codon read-through, trans-splicing, and in vitro transcribed RNAs (extensively reviewed in ref. 23) Although several of these strategies have shown encouraging results in preclinical research and early clinical trials, further studies and trials are needed to fully understand the safety and efficacy of these approaches and determine their clinical applicability. Hence, there still is an unmet need for the patients, warranting research into novel, especially systemic, therapeutic strategies.

We here studied AON-mediated exon skipping as systemic therapeutic approach for RDEB. The potential of AON-mediated exon skipping is underscored by clinical studies in several genetic diseases,[28] but it is most advanced in Duchenne muscular dystrophy. A previous study by Turczynski *et al.*, investigating exon skipping in *COL7A1*, showed exon skipping in vitro,[17] whereas Goto *et al.* showed exon 70 skipping and restoration of type VII collagen expression 16 hours after a single local injection directly into a transplanted skin equivalent on the back of rats.[19] However, the observed exon skipping efficiency was rather low in the latter study as

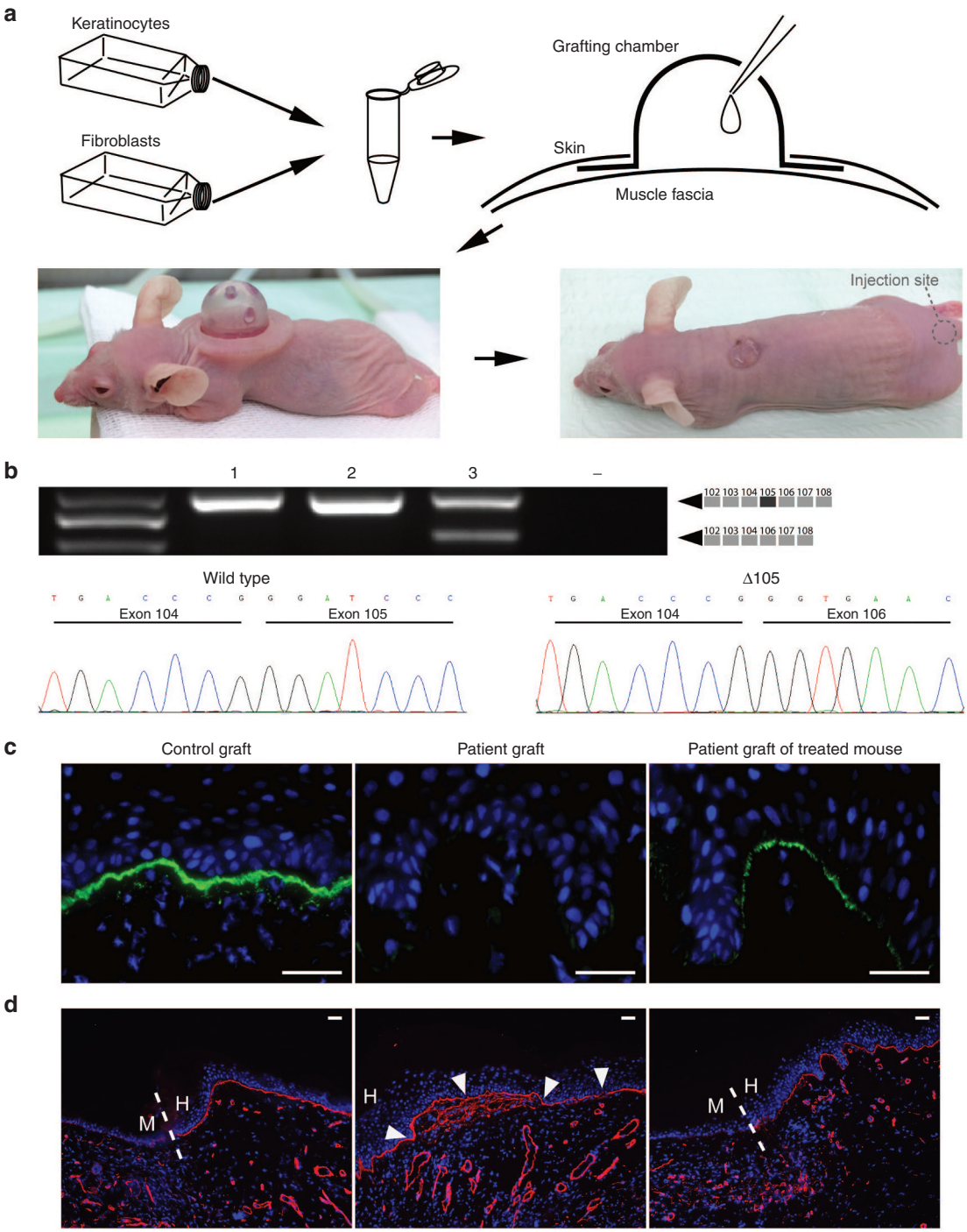

Figure 3 ***In vivo*** **antisense oligonucleotide (AON)-induced exon skipping leads to restoration of type VII collagen synthesis upon systemic treatment.** (**a**) Illustration of the skin-humanized mouse model. Primary control keratinocytes and fibroblasts were seeded into silicone grafting chambers implanted on the back of athymic nude mice. The injection site is indicated (grey dotted circle). (**b**) RT-PCR showed *in vivo* exon 105 skipping after eight weeks of treatment. Saline treated healthy and patient skin grafts (lane 1 and 2, respectively) show only a wild-type RNA product including exon 105, whereas patient skin grafts from specific AON-treated mice revealed skipping of exon 105 (lane 3). Sanger sequencing confirmed skipping of exon 105. (**c**) IF staining on cryosections of grafts from mice treated as in a, revealed *de novo* expression of type VII collagen (green) in patient grafts grown on AON-treated mice. Type VII collagen expression varied along the BMZ and the overall amount of protein expression was reduced compared to the control graft. (**d**) Staining for type IV collagen (red), reveals widening and off shoots of the basement membrane zone in untreated patient skin grafts, indicated by white arrows. This widening is neither observed in healthy control nor treated patient skin grafts. Dotted line indicates graft border, and human (H) and mouse (M) epidermis is indicated. Scale bar = 50 μm.

shown by RNA and type VII collagen expression analyses. Further, during the revision of this manuscript another study on exon skipping for *COL7A1* was published.[29] Turczynski *et al.*, showed *in vivo* restoration of type VII collagen expression and anchoring fibrils in a human skin graft mouse model after one or two local injections with AONs targeting exons 73 and 74, or 80. *In vitro,* 20% of patient keratinocytes regained type VII collagen expression. By thorough *in silico* selection of AONs, we were able to achieve high exon skipping efficiency at the RNA level, and restoration of protein expression *in vitro* in 50% of patient keratinocytes, at a total level of approximately 14% compared to control cells. The intensity of type VII collagen staining of treated patient cells positive for type VII collagen was comparable to healthy control cells.

Subsequently, we elaborated on the clinical relevance of AON-mediated exon skipping for RDEB by investigating the effect of systemic AON administration on patient skin grown on the back of nude mice. Systemic treatment is highly preferred for the RDEB-gen sev patient population, as RDEB also affects the internal lining of several organs, such as the esophagus and genital mucosa.[1] Our *in vivo* data demonstrate, for the first time, that AONs administered systemically by subcutaneous injections induce exon skipping and restoration of type VII collagen protein synthesis at a distance from the injection site, providing proof-of-concept for AON-mediated exon skipping as a systemic treatment for RDEB.

Separately, we have studied the effect of exon skipping on the functionality of type VII collagen.[19] Type VII collagen lacking the amino acids encoded by exon 105 showed conserved functionality in various biochemical and *in vitro* cell assays. Triple helix thermostability, fibroblast migration and adherence were all comparable to wild-type type VII collagen protein. Moreover, upon injection of type VII collagen lacking exon 105 in a type VII collagen hypomorphic mouse model, normal incorporation in the basement membrane zone was observed. Our observations that the newly formed type VII collagen lacking exon 105 is normally incorporated in the graft's BMZ, whereas it is known that several dominantly inherited mutations cause aberrant type VII collagen deposition patterns at the BMZ,[30] further supports the lack of strong functional consequences of exon 105 skipping.

It is yet unknown which cell type is responsible for the restoration of type VII collagen expression upon AON treatment: keratinocytes, fibroblasts, or both. Notably, a higher number of basal keratinocytes show expression of type VII collagen, compared to dermal fibroblasts, as can be also seen in **Figure 2d**. Therefore, the treatment effect is anticipated to be higher if the systemically administered AONs reach the basal keratinocytes. Targeting only the dermal fibroblasts may, however, already result in significant amelioration of the phenotype, as indicated by the clinical improvement seen after injections with type VII collagen expressing fibroblasts.[25] Moreover, as shown by fibroblast injections in type VII collagen hypomorphic mice followed by type VII collagen expression and skin integrity analyses, 30–35% of type VII collagen expression levels are sufficient to prevent skin fragility.[31] In combination with the strong type VII collagen expression—phenotype correlation,[16] complete restoration of type VII collagen expression seems not a prerequisite for successful exon skipping with significant phenotypic improvement.

AON-mediated exon skipping is preeminently a precision medicine approach, which is especially true for the *COL7A1* gene, where most mutations are scattered throughout the entire 118 exons. However, looking at the *COL7A1* mutation database of more than 670 published mutations in over 1,000 DEB patients (www.deb-central.org), 70–75% of all RDEB mutations are located in in-frame exons and almost 40% are located in exons encoding Gly-X-Y motifs only.[32–34] Treating all these patients would thus require an AON-library targeting most (if not all) of the 107 in-frame *COL7A1* exons. As each AON is considered a new drug, this will pose challenges in, for instance, trial design due to the limited number of patients. However, in case one or two medicinal AONs for RDEB would have obtained marketing authorization, this would allow discussions with the regulators on extrapolation of data on efficacy and safety for AONs with identical chemistries,[35] thereby facilitating the development of AONs for a larger group of patients with RDEB.

Translation into the clinic will undoubtedly come with challenges. A lot can, however, be learned from studies performed in Duchenne muscular dystrophy where AON-mediated exon skipping with 2'-*O*-methyl phosphorothioate AONs has now been tested in more than 300 patients.[36–38] No serious adverse effects have been noted that would preclude its clinical application and the AON treatment is generally well tolerated. However, transient proteinuria and thrombocytopenia occur more frequently in AON than placebo-treated cohorts.[38] Subcutaneously injected oligonucleotides cause injection site reactions like redness, irritation and induration. In RDEB, where the skin of the patient is severely affected and fragile and injection site reactions might worsen the disease, intravenous administration might therefore be the preferred delivery route of the AONs.

In conclusion, this study provides strong proof of concept for systemic treatment of generalized severe recessive dystrophic epidermolysis bullosa by AON-mediated exon skipping.

Materials and methods

Ethics statement. Informed consent was obtained before the use of healthy control and patient skin according to the Declaration of Helsinki Protocols. The institutional animal care and use committee approved the use of all experimental animals for this study. The mice were housed in a clean facility and provided with water and nutrition *ad libitum*.

Cell culture. Control keratinocytes and fibroblasts were isolated from skin after informed consent of healthy patients that underwent reconstructive surgery. RDEB patient keratinocytes and fibroblasts were isolated from a biopsy after informed consent of a patient having the homozygous c.7828C>T, p.Arg2610Ter null mutation in exon 105 of the *COL7A1* gene. After incubation of the skin in trypsin (Invitrogen, Carlsbad, CA) for 1 hour at 37 °C 5% CO_2, the epidermis sheet was separated from the dermis with tweezers. Subsequently, the epidermis was cut into small ~1 × 1 mm pieces followed by a 5 to 10 minutes incubation in trypsin (Invitrogen) at 37 °C for separation. Bovine calf serum

(BCS) (Gibco, Life Technologies, Bleiswijk, the Netherlands) was added to the solution to stop trypsinization. The cells were pelleted by centrifugation for 10 minutes at 200 g and resuspended in complete Cnt-07 (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) serum-free medium to be plated into a culture petri dish. For continuation of culture, the cells are split in 3 when 90% confluence was reached. The dermis tissue obtained after the first trypsinization step described above, was cut into small pieces and spread to the bottom of a culture petri dish. Culture medium was daily added drop-wise to prevent floating of the tissue. Once the tissue-surrounding area was confluent with fibroblasts, the tissue was removed from the petri dish and the monolayer cells were harvested and seeded into a new petri dish. For continuation of culture, the cells were split in 3 when 90% confluence was reached. For the culture of fibroblasts, a mixture with a 6:4 ratio of F-12 nutrient (Gibco) (completed with 10% BCS (Gibco), glutamin (Invitrogen), streptomycin (Invitrogen), and penicillin (Invitrogen)) and Amniochrome (Lonza, Cologne, Germany) was used.

Antisense oligonucleotides. Two specific AONs were used to induce skipping of exon 105 of the *COL7A1* gene. AON1, 5'-GAUACCAGGCACUCCAUCCU-3', and AON2, 5'-CAUGAA GCCAACAUCUCCUU-3'. A nonspecific AON 5'-GCUUUU CUUUUAGUUGCUGC-3' was used as negative control and with the addition of a 5'-FAM 537.46 fluorescent label as positive control. All AONs comprise 2' *O*-methyl modified bases and phosphorothioate linkages, and were synthesized and purified by reverse-phase high-performance liquid chromatography (Eurogentec BV, Liège, Belgium).

In vitro transfection. In vitro cationic lipid transfection experiments were performed in 12-well plates using polyethylenimine (PEI) (MBI Fermentas, Life Technologies, Bleiswijk, the Netherlands) and Lipofectamine-2000 (LF) (Invitrogen). The lipid-AON complex formation was optimized to a weight:weight ratio of 1:1 for both PEI and LF. PEI was used to transfect fibroblasts and LF was used to transfect keratinocytes. Prior to transfection, cells were grown to 70–80% confluency, washed, and fresh medium was added to the wells. For the transfection using LF, the medium was replaced with Opti-MEM (Gibco). Lipid-AON complexes were formed according to the manufacturer's protocol and drop-wise added to the cells at a final concentration of 250 nmol/l of AON in the medium. After six hours of incubation at 37 °C 5% CO_2 the medium was removed, cells were washed, and complete culture medium was added.

In vitro RNA and protein analysis. For the analysis of exon skipping on RNA level, RNA was isolated 48 hours after transfection using RNeasy Micro Kit (Qiagen, Hilden, Germany). Medium was removed from the wells prior to the adding of the lysis buffer (provided by the kit). A cell scraper was used to help lyse the cell monolayers. The lysate was collected in a 1.5 ml tube, vortexed for 1 minute, flash frozen in liquid nitrogen, and stored at –80 °C before RNA isolation according to the manufacturer's protocol. Subsequently, RNA was reverse transcribed using Superscript-III (Invitrogen) reverse transcriptase. Reverse transcription was followed by PCR analysis of exon 105 of the *COL7A1* gene using primary (forward 5'-TCAGCTGTGATCCTGGGGCCT-3'; reverse 5'-AGGGCAGCAAGGGAGAGCCT-3') and nested (forward 5'-AGGGCAGCAAGGGAGAGCCT-3'; reverse 5'-TTTGT-GTCCTGCCAGCCCGG-3') primers.

For the analysis of type VII collagen expression, cells were grown on glass coverslips (Menzel-Gläser, Braunschweig, Germany) in 12-well plates. The culture medium was removed from the wells 72 hours after transfection and the cells were washed followed by fixation using an ice-cold 1:1 methanol-acetone solution followed by air-drying and storage in -20°C. IF staining was used to visualize type VII collagen. The cells were stained using LH7.2 (gift prof. dr. I.M. Leigh) primary and goat anti mouse Alexa488 labeled secondary antibody as described.[16] Twenty microliters of cell lysates were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto 0.45 µm nitrocellulose membranes (Millipore, Amsterdam, the Netherlands). Membranes were blocked with blocking buffer (50 mmol/l Tris-HCl pH 7.4, 150 mmol/l NaCl, 0.05% Tween-20 with 5% non-fat milk). Blots were stained using the rabbit anti collagen VII antibodies LH7.2 in blocking buffer overnight at 4 °C. Horseradish peroxidase-conjugated goat anti-rabbit antibody was added for 1 hour. Enhanced chemiluminescence prime reagent (GE Healthcare, Chicago, IL) was used to develop blots and pictures were captured using a Fusion SL system (Peqlab, Darmstadt, Germany). For dot blot analysis, 200 µl of conditioned medium were immobilized on a 0.45 µm nitrocellulose membrane using a Bio-Dot microfiltration apparatus (Bio-Rad, Hercules, CA). The membranes were blocked with 5% (w/v) skim milk powder (Applichem, Darmstadt, Germany) in Tris-buffered saline with 0.1% (v/v) Tween-20 (Sigma-Aldrich, Saint Louis, Missouri, USA) for 30 minutes at RT, and incubated with the polyclonal LH7.2 antibody dissolved in blocking buffer for 30 minutes at RT. After washing three times for 5 minutes with Tris-buffered saline, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 30 minutes at RT. Blots were developed as for Western blots and data were recorded with the Fusion software.

Generation of human skin grafts. For the generation of skin grafts, a mouse model was used as described.[21] Briefly, primary cultured fibroblasts and keratinocytes were used to reconstitute human skin on the back of Atymic nude mice (Charles River strain 490). For the validation of the mouse model, four mice were grafted using healthy control cells. After validation, six mice were grafted with RDEB patient cells. After implantation of the silicone grafting chamber, a mixture of 6×10^6 fibroblasts and 6×10^6 keratinocytes was seeded in the grafting chamber in a total volume of 400 µl in 1% low calcium BCS (HyClone) Dulbecco's Modified Eagle's Medium (DMEM) (Gibco). Prior to use, the BCS was chelexed using Chelex-100 (Bio-Rad) resin to remove calcium ions. Eight days after implantation, the silicone grafting chamber was removed and the wound was left to heal forming a scab in the process. Around 10 days postremoval of the grafting chamber, the scab fell off and the treatment phase was initiated.

Treatment of the patient mice. Once the scabs fell off, the treatment was started. The treatment scheme was composed of

five daily injections of 50 mg/kg of each AON in a 0.15M NaCl solution for 8 weeks. The subcutaneous injections were given in the trunk of the mice around 7 cm distal to the graft. Four mice with patient grafts were treated with the mixture of AON1 and AON2 solution, and two mice with patient grafts and two mice with control grafts were injected with saline solutions as negative controls.

In vivo RNA and protein analysis. One day after the last injection of 8 weeks of treatment, the mice were sacrificed and the skin grafts were harvested. The entire full skin thickness grafts were removed from the back of the mice and flash frozen using liquid nitrogen and stored at –80 °C for further analysis. For the analysis of exon skipping at the RNA level, a cryosection of 50 µm was cut on a Leica CM3050S cryostat and RNA was isolated, reverse transcribed, and PCR was performed as described above. For the analysis of human type VII collagen expression, 4 µm cryosections were stained using Zenon (Thermo Fisher Scientific, Life Technologies) labeled LH7.2 monoclonal mouse-anti-human antibody. Cell nuclei were stained using Hoechst. Sections were analyzed using a Leica DMRA fluorescence microscope. Calbiochem polyclonal rabbit-anti-human antibodies were used for indiscriminative type VII collagen staining. Silenus monoclonal PHM12 mouse-anti-human was used for type IV collagen staining.

Supplementary material

Figure S1. Clinical pictures of RDEB patient with stop mutation in exon 105.
Figure S2. Validation of the skin-humanized mouse model.

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